AUTOMATED ANALYSIS OF MULTIPLE SECTIONS FOR THE DETECTION OF OCCULT CELLS IN LYMPH NODES

WE Mesker, FS Doekhie, H Vrolijk, R Keyzer, WCR Sloos, H Morreau, PS O’Kelly, GH de Bock, RAEM Tollenaar, HJ Tanke

Clinical Cancer Research 2003;9(13): 4826-4834

This study was financially supported by the “Anna en Maurits de Kock Foundation” and the Dutch Cancer Society (Grant 2000-2211).
ABSTRACT

Background: At present, reverse transcription polymerase chain reaction (RT-PCR) against carcinoembryonic antigen mRNA is one of the few research tools for the detection of occult cells in histopathologically assessed negative lymph nodes from patients with colorectal cancer. The aim of this study was to investigate the suitability of supervised low-resolution image analysis of immunohistochemically stained sections as alternative.

Patients and methods: Multiple sections (n = 50) of regional lymph nodes from patients with colorectal cancer were immunohistochemically stained and analyzed by applying low-resolution image analysis (flatbed scanning) for semiautomated detection of cytokeratin (CK)-positive stained cells. The sensitivity of this approach was demonstrated for 20 patients with stage II colorectal cancer and compared with RT-PCR regarding the detection of clinically assessed recurrence of disease within 10 years.

Results: CK+ cells were detected in all of the patients (n = 6; 100%) with recurrence, compared with five patients (83%) found positive by carcinoembryonic antigen RT-PCR. From patients (n = 14) who did not develop a recurrence, eight (57%) had positive lymph nodes. In all patients with recurrence, we visually identified at least one group of CK+ cells (>2 cells).

Conclusions: Automated image analysis is a promising tool for the detection of occult cells in histopathologically negative nodes. It is potentially more sensitive but less specific for detecting recurrence of disease than conventional histopathology or RT-PCR and is particularly useful for the evaluation of sentinel nodes. Furthermore, it opens new ways for basic research of occult cells based on molecular profiling after laser-microdissection.

INTRODUCTION
The presence of lymph node metastasis is one of the most important prognostic factors and therapeutic selectors for many types of cancer. Despite the prognostic value of lymph node status as assessed by conventional histopathology, a significant percentage of patients with node-negative colorectal carcinoma (diagnosed as lymph node negative by conventional examination) develop recurrence of disease.\textsuperscript{1-3} Reasons for such exceptions are the biology of the tumor but also the limited sensitivity of conventional histopathology to detect rarely occurring occult cells in lymph nodes. For practical reasons only a few hematoxylin and eosin (HE) stained sections are examined, which limits the sensitivity of the technique to detect occult tumor cells.
Recently, alternative approaches to detect occult cells in lymph nodes have been described. Liefers et al.\textsuperscript{1} examined lymph nodes from a group of 26 patients with stage II colorectal cancer, originally reported as negative by histopathology, using RT-PCR against carcinoembryonic antigen (CEA) messenger RNA. In their retrospective study, 14 of 26 patients were reported as positive for CEA. The 5-year recurrence-free survival for the CEA positive patients was 50% and for CEA negative patients, 91%. Others found similar results.\textsuperscript{2,3}

A different approach to increase sensitivity is by immunohistochemistry (IHC). This method relies on the analysis of IHC stained sections of lymph nodes for the detection of immunostained cells. It has been reported for patients with breast carcinoma that the application of IHC in combination with the analysis of multiple sections results in the detection of up to 35% more positive nodes as compared to conventional histopathology.\textsuperscript{4-12} The clinical value however of this observation needs to be confirmed.

The sensitivity of the detection of occult cells has been shown to increase with the number of sections per lymph node examined up till a certain number of sections,\textsuperscript{13} but the practical applicability of multiple sectioning is limited by the labor intensive nature of preparing a large amount of IHC stained sections. For the analysis of sentinel nodes, however, multiple sectioning is highly recommended by the Association of Directors of Anatomic and Surgical Pathology (ADASP) and considered practically feasible.\textsuperscript{7}

In this article, we describe an approach in which IHC staining and multiple sectioning is combined and subjected to novel high-throughput automated imaging. This imaging system uses a high performance flatbed scanner (FBS\textsc{c}), which is able to digitize, in one A3 format, hundreds of cytological or histological specimens. Digitally acquired images of the IHC stained tissue are then automatically analyzed for the presence of positive-stained occult cells. Storage of cell coordinates allows for direct morphological evaluation using conventional microscopy.

In this study, we have compared visual examination of cytokeratin-immunostained serial sections (as gold standard) with automated analysis and with the RT-PCR data from the referred Liefers article\textsuperscript{1} with the emphasis on the sensitivity of the method to detect patients with recurrence of disease within a period of 10 years after a diagnosis of colorectal cancer tumor-node-metastasis (TNM) stage II.

**PATIENTS AND METHODS**

**Patients**

From 20 patients with TNM stage II colorectal cancer, lymph nodes were obtained consecutively from curative resections performed at the Department of Surgery of the Leiden University Medical Center (LUMC) between January 1990 and February 1992. From this material, originally studied using RT-PCR by Liefers et al.\textsuperscript{1}, 119 of 246 lymph nodes were still available. Twenty-one blocks showed poor material not suited for analysis (fat tissue, degenerated material, no histological material remaining).
Preoperative and perioperative examinations of the patients showed no evidence of metastatic disease. Follow-up was carried out in accordance with the department’s protocol (Department of Surgery, LUMC) and was based on periodic evaluations of the patient. The follow-up of the patients was at least 10 years and was updated by checking the patient files as of 1 February, 2002.

**Sectioning and immunohistochemical staining**

In the original study, one-half of the node was fixed in formalin and was embedded in paraffin for routine histopathological examination. The other half of the resected node was used for RNA isolation for the analysis of CEA-specific mRNA using RT-PCR. For the present study, all of the available lymph nodes were analyzed for those patients who were originally PCR-positive for CEA. From the PCR-negative group for each patient, six lymph nodes were chosen randomly to match the average number of nodes in the positive group. From this material, serial sections (10 sections of 5 μm in series at each level) were cut with intervals of 200 μm until no material was left in the paraffin block. Sections were stained for cytokeratin (CK) using the antibody AE1/AE3. The sections were hydrated and subjected to sodium citrate (0.01 M, pH 6.0 at 100°C) for 10 min before incubation with the mixture of primary biotinylated monoclonal anti-cytokeratin antibodies AE1/AE3 (Dako, Glostrup, Denmark). Immunostaining was based on the avidin-biotin-peroxidase technique using 3,3’-diaminobenzidine (DAB) as the endpoint product; all of the sections were counterstained with hematoxylin.

**Method of analysis of the slides**

All of the IHC slides were first manually examined in a very thorough way. The results served as the gold standard for the automated analysis. A node was called positive when at least one IHC positive cell was found (excluding white blood cells, macrophages e.g. known for nonspecific staining) confirmed by a second person (a pathologist). Subsequently sections were recorded using the flatbed scanner. Automated analysis was performed on all of the recorded nodes. The location of all manually detected CK+ cells was marked on printouts of the recorded images and compared with the automated analysis.

**Automated analysis**

The system consists of a flatbed Agfa XY-15 scanner interfaced to a 933 MHz Power Mac G4 computer via a SCSI-2 interface. The optical resolution was 5000 dpi in both directions corresponding to a pixel distance of 5 μm image acquisition. A special mold has been constructed to scan a maximum of 45 microscopic slides automatically (Figure 1). Digitization was performed using the ColorExact software package from Agfa. In addition to the system software, we designed dedicated image analysis software to analyze the bed-scan for the presence of microscopic slides within the mold and the lymph node sections on each slide. The analysis of the lymph node sections was divided into the following steps:
the selection of a lymph node, the detection of candidate occult cells, and the measurement of cell features (area, shape, peak intensity, and averaged probe color). On the basis of these features, eventual falsely selected objects (i.e. other than CK+ cells) were recognized as such. The analysis of a lymph node section resulted in a gallery of images of the candidate occult CK+ cells found and an overview image of the lymph node with position markers where the events were found (Figure 2). On the basis of the gallery, the majority of falsely selected objects was easily recognized visually. When needed, the stored cell coordinates were used to relocate events by automated microscopy for visual interpretation at high spatial resolution (Figure 3). The total time necessary to automatically process a lymph node, which involved 80 slides with 5 sections per slide, was 81 minutes, of which 1 minute was required to make a full bed-scan at lower resolution and to determine the coordinates of the image crops for the scanning of the individual sections.

For the optimization of the selection algorithm of the automated analysis program, a positive node was analyzed. Parameters were set on the detection of groups and single CK+ cells. Recorded images were automatically analyzed, and the results were compared with those obtained by conventional microscopy. Using optimized selection criteria for automated analysis, we detected 34 (94%) of 36 visually recognized cells by automated analysis. These algorithms were used for the present study.

**Figure 1.** A3 size mould to hold 15 x 3 = 45 slides on the AGFA XY15 scanner. Each slide may contain several tissue sections.
Statistical analysis
We have reanalyzed the set of patients previously investigated by Liefers et al.\(^1\) because of the availability of the material and the long-term follow-up (10 years). Obtained results using our method (IHC-automated analysis) were compared with the updated data of the RT-PCR study on the same set of patients. Carefully performed manual examination of the same slides served as the gold standard for this comparison. First, the automated analysis was compared with the manual evaluation. The data were described comparing patients with a recurrence of disease versus nonrecurrence, regarding IHC-automated analysis (Table 1) and RT-PCR (Table 2). Then, IHC was compared to RT-PCR by calculating the sensitivity and specificity (Table 3). Finally, we calculated the optimal distance for the sectioning of the paraffin material to detect all of the patients with a recurrence of disease (Figure 4).

RESULTS
IHC-automated analysis compared to conventional microscopy
A total of 119 lymph nodes (from 20 patients) were available; 33 were found positive and 65, negative, and 21 were not analyzed (see "Patients and Methods"). Per lymph node on average, 49 (range, 8-81) histological sections were analyzed. Comparing automated analysis with visual analysis using conventional microscopy revealed that, from a total of 33 visually evaluated positive nodes, two nodes were missed using automated analysis. Both nodes contained only one cell resulting in a sensitivity of 94% to detect a positive node. However, both patients had three more nodes in which occult cells were detected. Therefore, no positive patients were missed by automated analysis. One patient was missed with visual analysis but was found positive on visual verification of the candidate cells after automated analysis. This case illustrates the imperfectness of conventional screening compared with an automated performance of the image analysis procedure. Table 4 presents the results of the lymph node analysis and the clinical outcome of the 20 patients.

IHC-automated analysis compared with RT-PCR
All of the patients (n = 6) who had developed a recurrence of disease were detected by IHC-automated analysis (Table 1). From 43 analyzed nodes in this patient group, 17 (40%) were found positive for CK-stained cells with on average eight cells per positive node. In all of these patients, groups of cells (number cells \(\geq\)2) were visually recognized.
From all other patients (n = 14) who did not develop recurrence of disease, 8 (57%) were found positive. In 16 (21%) of 77 nodes analyzed in this group, CK+ cells were found. Only five patients (36%) had cells located in groups. One patient (7 CK+ groups) died within 1 month after operation because of a gastric hemorrhage. One patient died from a cause other than disease, and three patients showed no evidence of disease 10 years after surgery.
In three patients, no groups of cells were detected. Using the RT-PCR method, we detected five (83%) patients with recurrence of disease. Forty-five nodes were analyzed in this group, of which 12 (27%) were found positive for CEA expression. Three patients (21%) with no recurrence of disease had positive lymph nodes (Table 2).

### Table 2. Results of RT-PCR for CEA expression (same patients as shown in Table 4)\(^a\)

<table>
<thead>
<tr>
<th>Disease status</th>
<th>Recurrence (n=6)</th>
<th>Non-recurrence (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive patients</td>
<td>5 (83%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>Positive lymph nodes</td>
<td>12/45 (27%)</td>
<td>5/109 (5%)</td>
</tr>
</tbody>
</table>

CEA, carcinoembryonic antigen;\(^a\) Data from Liefers et al.\(^{1}\)

### Sensitivity and specificity

Realizing that a relatively low number of patient samples has been investigated, we, nevertheless, calculated the sensitivity and specificity of both methods. The sensitivity of IHC-automated analysis to detect CK+ nodes in patients with recurrence of disease was 100%; the specificity was 43%. The sensitivity and specificity of the RT-PCR method on the same set of patients studied by Liefers et al.\(^{1}\) are, respectively, 83% and 79% (Table 3).

### Table 3. Calculation of the sensitivity and specificity of IHC-automated analysis and RT-PCR with respect to the detection of patients with recurrence of disease

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC-automated analysis (cut-off level: 1 \text{ CK}^+\ \text{cell})</td>
<td>100%</td>
<td>43%</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>83%</td>
<td>79%</td>
</tr>
</tbody>
</table>

### Sampling distance of paraffin blocks

By reanalyzing the data, we calculated the effect of the distance of sectioning of the paraffin material with respect to successful detection of recurrence of disease. \(\text{Figure 4}\) shows the effect of varying the distance between sections with respect to
Chapter 4

Figure 2. A. Gallery with images of candidate occult cells detected in a lymph node section of a patient with colorectal carcinoma. Selection of candidate cells is based on immunoperoxidase (brown) staining for cytokeratin. Counterstaining is performed with hematoxylin (blue). B. Overview of the lymph node with markers displaying the location of the candidate cells.

the detection of positive lymph nodes ("positive" was defined here as containing at least one CK+ cell). Additionally, the percentage of lymph nodes is shown in which at least one group of cells (≥ 2 CK+ cells) was found. The sensitivity of both the visual and automated detection decreases from 100% and 93% to, respectively, 98% and 85% when one-half of the number of sections is analyzed.
Automated analysis for detection of occult tumor cells in lymph nodes

Figure 3. A. Examples of images recorded by the flatbed scanner at 5600 dpi. Sections of lymph nodes from patients with colorectal carcinoma. Cells are positively stained for cytokeratin and counterstained using hematoxylin. B. Zoom function of recorded image; arrow marks single cytokeratin-positive cells (1b, 2b) or a small group of cytokeratin-positive cells (3b). C. Conventional microscope image of the same cells. Recorded with color CCD camera using x16 objective.

One CK+ lymph node containing one group (2 cells) was missed when only one in every two sections was analyzed. When the detection of at least one CK+ group of cells was used as the criterion, sensitivity decreased from 100% to 90% and from 93% to 73% for automated and visual inspection, respectively.
DISCUSSION

This study shows that visual examination of multiple IHC stained sections for the presence of occult tumor cells can be automated by low-resolution image analysis. As such, this method may serve as a useful alternative for RT-PCR, particularly when large numbers of sections are analyzed (at least 50 per node for this study). In all of the patients ($n = 6$) with recurrence of disease, IHC positive cells were detected using this method, demonstrating its feasibility for this application. Compared with the RT-PCR study previously performed by Liefers et al., automated analysis of IHC stained sections appeared to be more sensitive in detecting patients with recurrence of disease (100% vs 83%). We also found more positive nodes in this group (40% vs 27%). However, in 57% of the patients who did not develop a recurrence of disease, positive cells were also found, compared with 21% for the RT-PCR method. When the cut-off level was increased from one cell to two CK+ cells, the specificity increased from 43% to 57%. This makes automated analysis more sensitive but less specific than the referred RT-PCR method and, therefore, requires additional analysis of the detected positive cells.

The number of analyzed patient samples, however, is considered too small to conclude that the differences are significant and meaningful. Discrepancies with the RT-PCR results may be attributable to unavoidable sampling errors. Yasuda et al. analyzed six serial sections using IHC and detected micrometastases in 92% of patients with recurrence but also found a high percentage of positive patients in the nonrecurrent group (70%). When more specific markers than pan-cytokeratin are available as published by Izbicki et al. for esophageal cancer (BerEp4), the high rate of false positive findings can possibly be reduced, thereby increasing the specificity of the current assay.
Automated analysis for detection of occult tumor cells in lymph nodes

It is estimated that routine HE analysis only has a 1% chance of identifying a focus of cancer cells less than three cells in diameter.\textsuperscript{17} This level of sensitivity implies the finding that about 25% of patients with colorectal cancers who are node negative by routine HE examination may develop distant metastases.\textsuperscript{1-4} The histopathological criteria for occult metastases are far from clear. Most of the studies identifying occult tumor cells have been performed in breast cancer. For instance, Turner \textit{et al.}\textsuperscript{18} and Kell \textit{et al.}\textsuperscript{19} report that, for patients with breast cancer with minimal axillary involvement, the presence of efferent vascular invasion or nodal hilar tissue invasion and the location of a micrometastasis in sinusoidal rather than parenchymal tissue may indicate a less favorable prognosis. Others have suggested the inclusion of the size of the metastasis, groups \textit{versus} single cells, and the microanatomical location of occult cells as prognostic features.\textsuperscript{20} However, evaluation of the potential value of these parameters has not been systematically pursued.

\textit{Table 4. Detection of micrometastasis and outcomes of patients with stage II colorectal cancer}

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Lymph nodes</th>
<th>Positive</th>
<th>Vital status</th>
<th>Disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>3</td>
<td>Dead</td>
<td>Recurrence\textsuperscript{a}</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1</td>
<td>Dead</td>
<td>Recurrence</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>Dead</td>
<td>Recurrence</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
<td>Dead</td>
<td>Recurrence</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>4</td>
<td>Dead</td>
<td>Recurrence</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>6</td>
<td>Dead</td>
<td>Recurrence</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>0</td>
<td>Dead</td>
<td>Other\textsuperscript{b}</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>0</td>
<td>Dead</td>
<td>Other</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>0</td>
<td>Dead</td>
<td>Other</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>2</td>
<td>Dead</td>
<td>Other</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>3</td>
<td>Dead</td>
<td>Other</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>0</td>
<td>Dead</td>
<td>Other</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>0</td>
<td>Alive</td>
<td>NED\textsuperscript{c}</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>4</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>0</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>1</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>17</td>
<td>10</td>
<td>2</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>2</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>19</td>
<td>6</td>
<td>1</td>
<td>Alive</td>
<td>NED</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>0</td>
<td>Alive</td>
<td>NED</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Recurrence death from local or distant recurrent disease; \textsuperscript{b} Other death from a cause other than cancer; \textsuperscript{c} NED, no evidence of disease.
Although RT-PCR methods have been investigated to improve sensitivity, specific markers still do not exist for many tumors. RT-PCR is proven to be very sensitive and able to detect 1 in 10 million cells but is often prone to false positive classification because of contamination, and illegitimate low-level expression of marker transcripts in normal lymph nodes has also been reported. Furthermore, PCR has the restriction that detected events cannot be morphologically evaluated and confirmed. Microscope-based analysis has the advantage of enabling morphological analysis of the detected occult cells by the pathologist. The use of monoclonal antibodies to further characterize the detected cells (either by bright-field or fluorescence microscopy) may be useful. It is evident that the number of positive nodes will increase on analysis of more sections, and may strongly increase. Automated analysis as described here may be further improved by connection of the scanner to an off-line automated microscope for rapid relocation of the detected events and by fine tuning of the cell classification algorithm with respect to the accuracy. The analysis is rather time consuming, and speed can be increased but is, when focusing on sentinel nodes, not a prerequisite. For use in a clinical setting, the sectioning and staining of all resected lymph nodes is too labor intensive. However, for the analysis of sentinel nodes, which most of the time involves only one to three lymph nodes, serial sectioning is highly recommended by the ADASP and is practically feasible.

The clinical significance of immunohistochemically detected tumor cells present in excised lymph nodes in case of colorectal cancer remains unclear. In a recent multi-institutional study of 736 patients with breast cancer, the presence of immunohistochemically detected occult cells in axillary lymph node metastases was found to be significant and, in case of postmenopausal women, was an independent predictor of overall survival. For colorectal cancer, this reaffirms the need for larger studies with longer follow-up.

The relatively large data set (4569 sections of 119 nodes of 20 patients) of the presented study allowed to examine the effects of the sectioning density on the detection of CK+ cells in a particular lymph node. It appeared that the number of CK+ cells decreased inversely proportional to the sampling distance (data not shown). This relation suggests that the CK+ cells are more or less randomly distributed throughout the node. Because the presence of CK+ cells was rather low in a number of CK+ lymph nodes, a large proportion of the lymph node has to be analyzed to classify the node as positive. As can be seen in Figure 4, one CK+ lymph node containing only two CK+ cells (group) was missed (even visually), when only one in every two sections was analyzed. When the detection efficiency of lymph nodes with at least one CK+ group is considered, one would expect that detection of groups, being larger than single cells, would be less dependent on the sectioning distance. Figure 4 shows the opposite, however. This can be explained by the low frequency of groups present in most of the lymph nodes when compared with the number of single CK+ cells and by the fact that most of those groups consisted of only 2 to 3 cells.
To better understand the biology of metastasis, research is needed to further characterize the detected cells, which may ultimately lead to an increase in specificity and diagnostic accuracy. Such information can be obtained by physical isolation of these cells by laser microdissection followed by single-cell RT-PCR and analysis of gene composition.24

Such research has recently been published by Klein et al.25, who found a different genetic make-up for single cells versus groups of cells. The clinical importance of these findings, however, is not yet known.

This information can be used to produce specific markers for diagnostic assays that may ultimately allow the identification of biologically important populations of cells that can be directly linked to clinical outcome.

The present study was performed on a well-documented selection of colorectal cancer patients and served as a model. It is obvious that a similar approach is also indicated for breast cancer or for melanomas in which lymph node involvement and, particularly, the role of the sentinel node is an important focus of research.8;9;26-31

REFERENCES


31. Statius Muller MG, van Leeuwen PA, de Lange-De Klerk ES et al. The sentinel lymph node status is an important factor for predicting clinical outcome in patients with Stage I or II cutaneous melanoma. Cancer 2001;91:2401-2408.